J Biol Phys (2007) 33:389–397
DOI 10.1007/s10867-008-9080-5

ORIGINAL PAPER

Electro-Optical Properties Characterization of Fish Type III Antifreeze Protein

Andrés G. Salvay · Javier Santos · Eduardo I. Howard

Received: 30 October 2007 / Accepted: 15 April 2008 /
Published online: 3 June 2008

Abstract Antifreeze proteins (AFPs) are ice-binding proteins that depress the freezing point of water in a non-colligative manner without a significant modification of the melting point. Found in the blood and tissues of some organisms (such as fish, insects, plants, and soil bacteria), AFPs play an important role in subzero temperature survival. Fish Type III AFP is present in members of the subclass Zoarcoidei. AFPIII are small 7-kDa—or 14-kDa tandem—globular proteins. In the present work, we study the behavior of several physical properties, such as the low-frequency dielectric permittivity spectrum, circular dichroism, and electrical conductivity of Fish Type III AFP solutions measured at different concentrations. The combination of the information obtained from these measurements could be explained through the formation of AFP molecular aggregates or, alternatively, by the existence of some other type of interparticle interactions. Thermal stability and electro-optical behavior, when proteins are dissolved in deuterated water, were also investigated.

Keywords Antifreeze proteins · AFPIII · Electro-optical behavior · Thermal stability

A. G. Salvay · E. I. Howard (✉)
Instituto de Física de Líquidos y Sistemas Biológicos (IFLYSIB), Universidad Nacional de La Plata,
c.c. 565, 1900BTE La Plata, Argentina
e-mail: howard@iflysib.unlp.edu.ar

A. G. Salvay · J. Santos
Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, R. Saenz Peña 352,
1876XD Bernal, Argentina

J. Santos
Department of Biological Chemistry and Institute of Biochemistry and Biophysics (IQUIFIB),
School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956,
1113AAD Buenos Aires, Argentina

1 Introduction

Antifreeze proteins (AFPs) are found in the blood and tissues of organisms that live in freezing environments, which is a product of their cold adaptation processes to defend their tissues from freezing injury. In these organisms, ranging from fish to bacteria, the effect of freezing is retarded, or the damage incurred upon freezing and thawing is reduced. AFPs can protect organisms from freezing by binding to ice and inhibiting its growth and recrystallization. The water solution of AFP exhibits a non-colligative freezing temperature depression. This is a result of its ice-binding ability, which inhibits the growth of embryo ice crystals that naturally emerged in the supercooling water [1].

This adsorption–inhibition mechanism operates at the ice surface to cause a nonequilibrium lowering of the freezing point below the melting point (thermal hysteresis). Adsorption requires a specific interaction between the ice-binding site of the AFP and a structural feature of the ice lattice. Both hydrophobic interactions and specific hydrogen bonds have been suggested to explain this binding [2–5]. It has been proposed that adsorbed AFPs restrict ice to growing between the bound proteins with a local surface curvature [6]. The increased surface area and curvature make it energetically less favorable for water to join the lattice and results in a local (nonequilibrium) depression of the freezing point. It has been suggested that cooperative interactions between AFPs on the ice surfaces are required for complete inhibition of ice crystal growth [6].

Although AFPs have been studied for more than 20 years, the precise mechanism by which they inhibit ice growth is still unclear. Unlike enzymatic proteins that can often be cocrystallized along with their ligands, there is no direct way to investigate the molecular interactions between AFPs and the growing ice crystals.

Because such a unique inhibitory function of AFP exhibits a high potential for cryoindustrial and cryomedical usages—such as cryopreservations of tissues and cells, improving storage of food, blood, tissues, and organs, cryosurgery, protecting crops from freezing, and maintaining the texture of frozen materials; it becomes more and more significant to achieve a detailed understanding of its ice-binding mechanism.

Fish Type III AFP (AFPIII; one type of fish antifreeze protein) is present in members of the subclass Zoarcoidei. AFPIII is a small 7-kDa—or 14-kDa tandem—globular protein with a compact structure of dimensions 24×26×40 Å in which the overall fold comprises numerous short β-strands and one turn of α-helix [7, 8]. It has been reported that the protein surface presents a dominant hydrophilic and a dominant hydrophobic region [9, 10], which results in the self-assembly of proteins when concentrations reach a critical aggregation concentration value (CAC) [9, 10].

The characterization of the interactions and the association state of AFPIII may be essential in order to understand the functional mechanism by which it inhibits ice growth.

In this work, we study the behavior of several electro-optical properties, such as low-frequency dielectric spectroscopy, circular dichroism (CD), and electrical conductivity at different protein concentrations. The difference in the behavior of the electrical conductivity above the concentration of 2.5 mg/ml may be due to the possibility that the molecules of AFPIII start to aggregate into some hydrophilic-like cluster, as has been reported [9, 10]. The dielectric permittivity spectrum of AFPIII at concentrations above this critical concentration shows the relaxation of a macromolecular dipole with an effective radius of 32 Å. Thermal stability and the behavior of electro-optical properties when proteins are dissolved in deuterated water were also investigated. In these studies, we observe different behavior when the protein is solubilized in D_2O.

2 Materials and Methods

The synthetic gene of the type III (isoform HPLC12) antifreeze protein from *Macrozoarces americanus* was received from the Peter Davies group and modified to adjust the sequence to the 1HG7 PDB entry. The protein was overexpressed in *Escherichia coli* and purified from inclusion bodies in a standard procedure [11]. After washing the inclusion bodies with Triton, the pellet was resuspended in a solution containing 2 M urea, 50 mM sodium acetate, pH 4.0. Then, the solution was spun at 20,000×*g* for 10 min, and the supernatant was charged on an SP column, washed, and eluted with a NaCl gradient.

The purified protein was dialyzed two times against distilled and deionized water and, then, lyophilized. Solutions of AFPIII at different concentrations were prepared by dissolution of the same lyophilized protein batch in H_2O and D_2O. The choice of working in pure and deionized water (either H_2O and D_2O) was made because other works related to the same protein were done in identical conditions [9, 10]. In this work, we use pure H_2O and D_2O, both with an electrical conductivity value of 1 μS cm^{-1}. The concentration of dilutions was determined from absorbance at 280 nm, using an estimated extinction coefficient from the amino acid sequence of 1,490 M^{-1} cm^{-1}, corresponding to absorbance per milligram per milliliter of 0.212.

Solutions and solvent densities were measured with a DMA 46 density meter (Anton Paar, Graz, Austria). Viscosity was measured using a digital Schott Geräte CT 1150 viscosity meter. Density and viscosity measurement of AFPIII solution in H_2O at the concentration used in this work do not show an appreciable difference from the values of density and viscosity of pure water.

Electrical conductivity was measured with a Radiometer CMD3 conductivity meter. The electrical conductivity σ of the sample can be written, on the basis of a simple additive rule, as $\sigma = \sum_i (|z_i|e)n_i u_i$, where each charged entity (protein, polyions, counterions, and ions) contributes through its concentration n_i, its charge $z_i e$, and its mobility u_i.

Dielectric measurements were made over a frequency range between 10^3 and 10^8 Hz using a Hewlett Packard LF4192A Impedance Analyzer. Raw data from biological samples usually include artifactual impedance at frequencies lower than 10^5 Hz due to electrode polarization [12]. This effect is more notable when the proteins are dissolved in high conductivity media. In order to minimize this effect, we use double-distilled and deionized water of electrical conductivity of 1 μS cm^{-1} as dissolving media. Electrode polarization effects at low frequency are corrected using the method proposed by Raicu et al. [13]. The dielectric permittivity spectrum $\varepsilon'(\omega)$ was determined by the relation $\varepsilon'(\omega) = K \cdot C_s(\omega)$, where $\omega = 2\pi f$ is the angular frequency, K is the cell constant, and C_s the capacitance of the sample.

CD spectroscopy measurements were carried out with a Jasco J-810 spectropolarimeter calibrated with (+)10-camphor sulfonic acid. Near-ultraviolet (UV) CD and Far-UV CD spectra were collected in pure water or in pure D_2O. Cells of 1.0 and 0.1 cm were used for near- and far-UV measurements, respectively. Data were acquired at a scan speed of 20 nm min^{-1} and at least five scans were averaged. Finally, blank (water) scans were subtracted from the spectra and values of ellipticity were generally expressed in units of degree squared centimeter per decimole. Final protein concentration ranged from 1.0 and 5.0 mg ml^{-1}, and, in thermal unfolding experiments, concentration was 0.1 mg ml^{-1}.

All measurements—except thermal stability experiments—were performed at 20°C.

3 Results

The conductivity measurements of AFPIII dissolved in H_2O and D_2O are shown in Fig. 1A and B, respectively. Changes in conductivity behavior around 2.5 mg/ml are related to the different mobility of the charges. This effect may be due to the formation of self assemblies of proteins around the CAC of 2.5 mg/ml. Another possibility for the explanation of conductivity behavior is the non-ideality of the AFPIII solution because of the existence of other types of interparticle interactions; in real solutions, even weak interparticle interactions will cause a concentration dependence of the observed properties—this is manifested as nonlinear behavior [14]. Comparing panels A and B in Fig. 1, we can observe a different performance of the electrical conductivity for the protein dissolved in H_2O and D_2O.

The dielectric method can bring valuable information for studies of the structure and dynamics of macromolecular solutions. This is because it can investigate the relaxation processes occurring in aqueous macromolecular solutions in an extremely wide range of characteristic times. The principles of measurement are very simple: the electrical

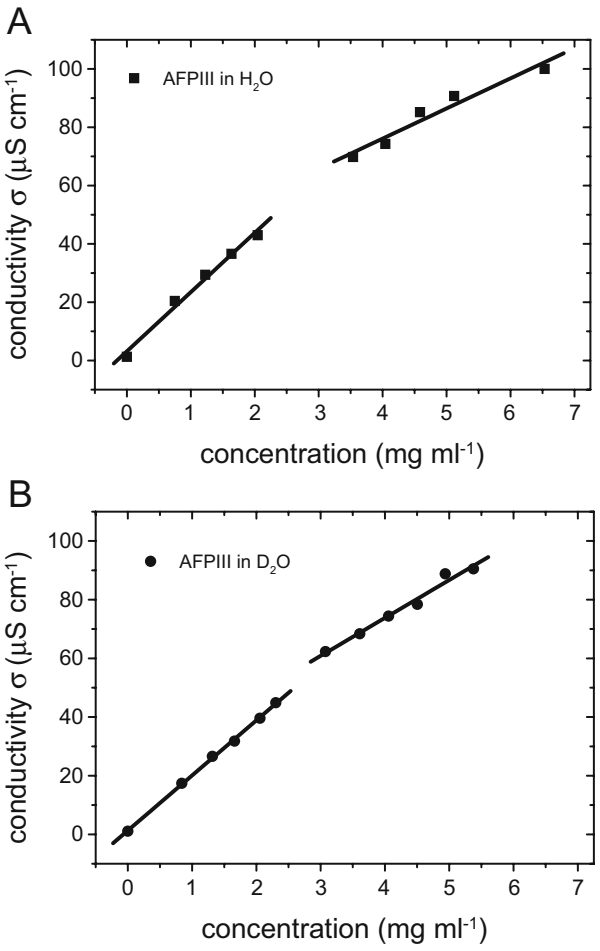

Fig. 1 Electrical conductivity σ measurements of AFPIII dissolved in: **A** H_2O and **B** D_2O

impedance of an appropriate device containing the sample to be investigated is measured as a function of the frequency f of the applied electric field. Figure 2 shows the low-frequency dielectric permittivity spectrum of AFPIII dissolved in H_2O at a concentration of 3.5 mg/ml. The spectra show the relaxation of a macromolecular dipole. The experimental data of the real part of the permittivity $\varepsilon'(\omega$; polarization per unit of the applied electrical field) is fitted with the Debye formula $\varepsilon'(\omega) = \Delta\varepsilon/(1+\omega^2\tau^2) + \varepsilon_\infty$, where $\Delta\varepsilon = \varepsilon_0 - \varepsilon_\infty$ is the dielectric increment, ε_0 the static permittivity, ε_∞ the infinite permittivity, and τ the dielectric relaxation time of the sample [15]. The best nonlinear fit parameters of the experimental data are: $\Delta\varepsilon$=3.23±0.15; τ=9.88 10^{-8}±1.04 10^{-8} s; and ε_∞=84.5±0.1. The size of the macromolecular dipole can be estimated using the determined relaxation time τ in the relation of Debye, $\tau = 4\pi a^3\eta/kT$, where η is the viscosity of the medium in which the macromolecule rotates—H_2O in this case, k is Boltzmann's constant, T is the absolute temperature, and a=32 Å the effective radius of the macromolecular dipole calculated when the macromolecule is considered to be spherical [16].

Figure 3 shows a ribbon diagram of AFPIII where the environment of the amino acid residue Y63 (d<4.5 Å) is represented in sticks. AFPIII was spectroscopically characterized to verify whether its tertiary structure was preserved in water, in conditions of virtually no ionic strength. AFPIII has only one aromatic residue, Tyr63, which presents it as an excellent probe to check the compactness and packing of side chains by near UV-CD spectroscopy. In particular, it tests the interaction between the C-terminal element of the chain and the rest of the molecule. It is important to note that Tyr63 has only 38.3 $Å^2$ of accessible surface area out of a total surface of 327.9 $Å^2$ (nearly 11.7%). The CD spectrum characterization of AFPIII is shown in Fig. 4. AFPIII in water showed a near UV-CD spectrum compatible with the presence of a defined tertiary structure. Clearly, 8.0 M urea obliterates the near UV-CD signals, showing that they are specific to tertiary structure. Concentration variations of AFPIII, ranging from 1 to 5 mg/ml, do not lead to a substantial change in its near UV-CD spectrum. This suggests that in the case of aggregate formation, this will not alter the Tyr63 environment. The particular shape of the far UV-CD spectrum of type III AFP was originally interpreted as a spectrum of an unstructured protein [17, 18]. In spite of this, the spectrum of type III AFP shows a native-absorption band between 215 and 230 nm, and it allows us to conduct unfolding experiments. It is important to note that the far UV-CD spectrum results are similar in shape to that obtained by other groups in the presence of salt [17, 19].

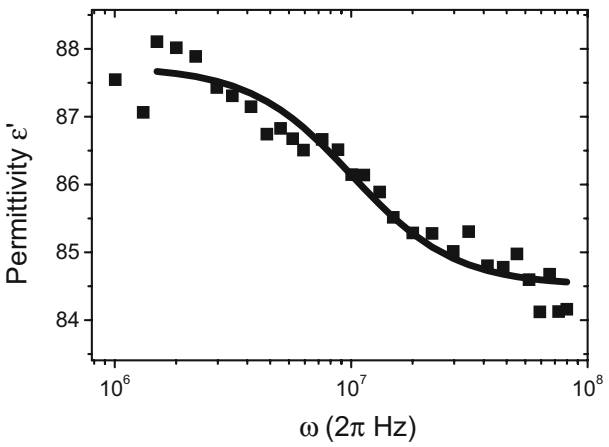

Fig. 2 Dielectric permittivity spectrum of 3.5 mg/ml of AFPIII in H2O. The *line connecting the experimental points* is the best fit with Debye formula $\varepsilon'(\omega) = \Delta\varepsilon/(1+\omega^2\tau^2) + \varepsilon_\infty$

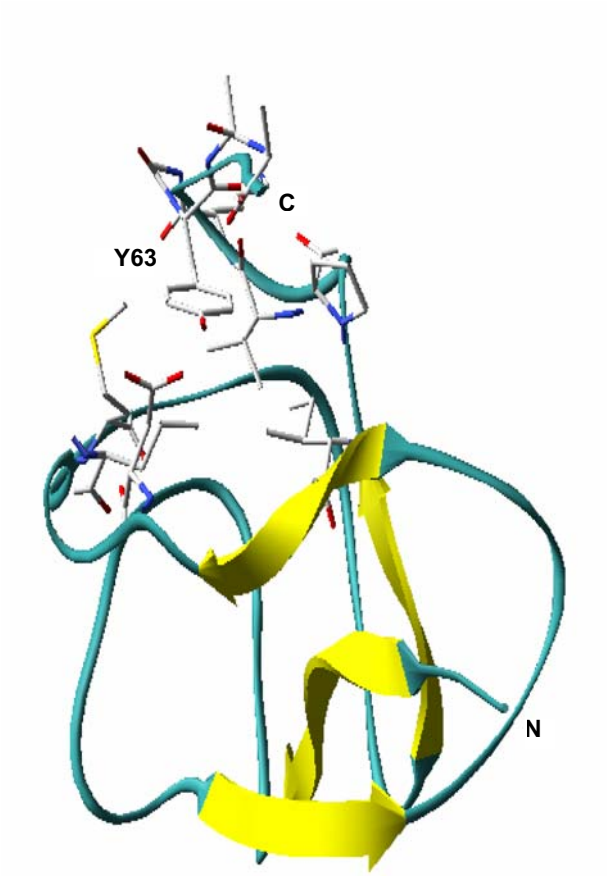

Fig. 3 Ribbon diagram of AFPIII. N- and C-terminals are indicated as *N* and *C*, respectively

Figure 5 shows the thermal stability of AFPIII in H_2O or D_2O at a concentration of 0.1 mg/ml. Thermal unfolding followed by far UV-CD of AFPIII in H_2O led to a remarkable increase in irreversible denaturation: near 70% of the native signal at 216 nm was recovered when the solution returned to the starting temperature. Interestingly, in D_2O, we recovered essential 0% of the far UV-CD signal, showing that the denaturation process is virtually irreversible in this condition. On the other hand, we observed in D_2O a significant increase in thermal stability (54.8°C vs. 50.5°C in H_2O).

4 Discussion and Conclusions

The results described in this work allow for the study of the interactions and association state of AFPIII in solution.

Electrical conductivity measurements of AFPIII dissolved in H_2O and D_2O show nonlinear behavior. Changes in conductivity behavior may be related to the different mobility of the charges around 2.5 mg/ml, or to the non-ideality behavior of the protein solution. If the changes of the conductivity are only associated with the mobility of the electrical charges, the responsibility for the conductivity behavior is the formation of

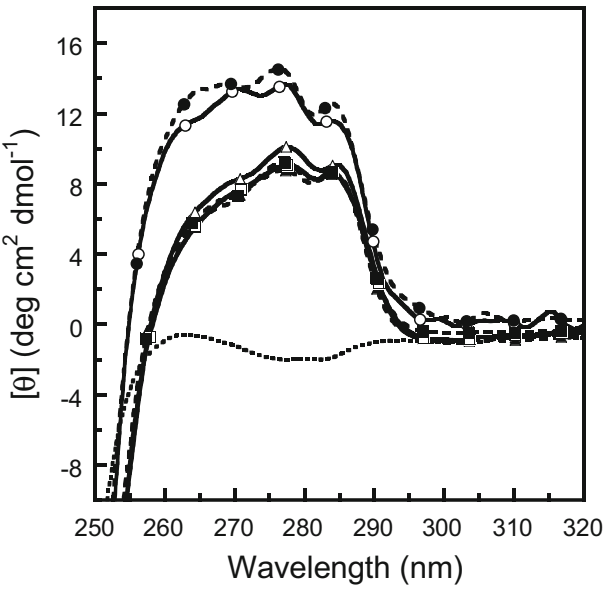

Fig. 4 Electrical dichroism spectrum of the AFPIII solutions: (—o—) 1.0 mg/ml H_2O; (—●—) 1.0 mg/ml D_2O; (—Δ—) 3.5 mg/ml H_2O; (—▲—) 3.5 mg/ml D_2O; (—□—) 5.0 mg/ml H_2O; (—■—) 5.0 mg/ml D_2O; (——) urea unfolded

self-assembly of proteins around the CAC of 2.5 mg/ml. The other possibility for the explanation of conductivity behavior is the existence of some other type of interparticle interactions [14]; in this situation, the positions, orientations, and movements of the particles are correlated, and this effect is manifested in the nonlinear relation between conductivity and concentration.

The dielectric spectrum of AFPIII in H_2O shows the relaxation of a macromolecular dipole. Dielectric measurements allow the determination of a relaxation time $\tau=9.88\ 10^{-8}\pm1.04\ 10^{-8}$ s for the dipole that corresponds to an effective radius of 32 Å considering a spherical macromolecular dipole. This macromolecular dipole may be attributed to the AFPIII

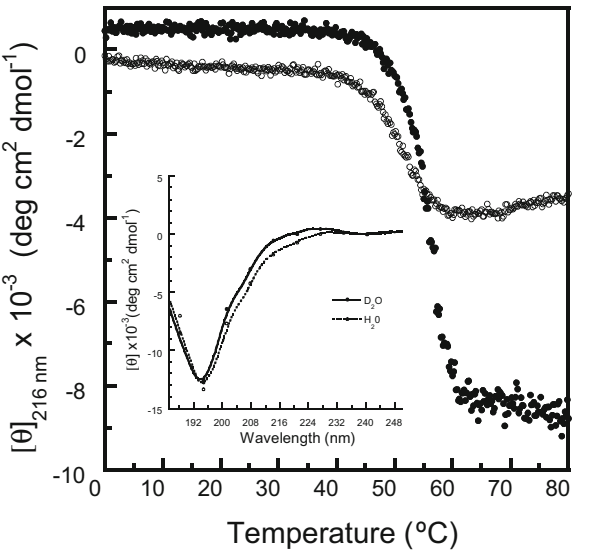

Fig. 5 Thermal unfolding of AFPIII in H_2O (*unfilled circles*) and D_2O (*filled circles*) followed by far UV-CD (inset)

aggregates. However, the form of the boundary between the water and the macromolecular dipole may mean that the appropriate viscosity is different from the bulk value [20]. The irregular nature of the protein surface may, for instance, introduce greater friction than appropriate for the ball-bearing representation on which dielectric relaxation model is based [20]; this would create a larger relaxation time than expected, and, in this case, the macromolecular dipole may be attributed to the AFPIII monomer and its hydration shell. From these results, both options are possible, and this question must await further investigations involving direct methods for the determination of the association state of the AFPIII.

Differences in the behavior of physical properties—such as electrical conductivity and thermal stability—were observed in this work when the Fish Type III Antifreeze proteins were dissolved in H_2O and D_2O. It has been proposed that the replacement of H_2O by D_2O decreases the solubility and increases the attractive intermolecular interactions of proteins [21]. These results suggest particularities of the hydration of AFPIII which, in turn, pose an interesting problem to address in further research.

Acknowledgments The authors thank A. Podjarny and A. Cousido from IGBMC, Strasbourg, J.M. Delfino from IQUIFIB, Argentina, J.R. Grigera from IFLYSIB, Argentina, Gabriel Koremblit, from UTN-Ushuaia, and the "Laboratorio de Ecofisiología" from CADIC, Ushuaia, Argentina for support and helpful discussion. The authors are very grateful to A.N. McCarthy from IFLYSIB for his critical reading of the manuscript. JS and EIH are members of the "Carrera del Investigador", CONICET. This work was supported by CONICET, UNLP, and UNQ.

References

1. Raymond, J.A., DeVries, A.L.: Adsorption inhibition as a mechanism of freezing resistance in polar fishes. Proc. Natl. Acad. Sci. U S A **74**, 2589–2593 (1977)
2. Cheng, Y., Yang, Z., Tan, H., Liu, R., Chen, G., Jia, Z.: Analysis of ice-binding sites in Fish Type II Antifreeze protein by quantum mechanics. Biophys. J. **83**, 2202–2210 (2002)
3. Jorov, A., Zhorov, B.S., Yang, D.S.: Theoretical study of interaction of winter flounder antifreeze protein with ice. Protein Sci. **13**, 1524–37 (2004)
4. Strom, C.S., Liu, X.Y., Jia, Z.: Antifreeze protein-induced morphological modification mechanisms linked to ice binding surface. J. Biol. Chem. **279**, 32407–32417 (2004)
5. Prabhu, N., Sharp, K.: Protein-solvent interactions. Chem. Rev. **106**, 1616–1623 (2006)
6. Wen, D., Laursen, R.A.: Structure-function relationships in an antifreeze polypeptide: the effect of added bulky groups on activity. J. Biol. Chem. **268**, 16401–16405 (1993)
7. Sonnichsen, F.D., DeLuca, C.I., Davies, P.L., Sykes, B.D.: Refined solution structure of type III antifreeze protein: hydrophobic groups may be involved in the energetics of the protein-ice interaction. Structure **4**, 1325–1337 (1996)
8. Ko, T.-P., Robinson, H., Gao, Y.-G., Cheng, C.-H.C., DeVries, A.L., Wang, A.H.-J.: The refined crystal structure of an Eel Pout type III antifreeze protein RD1 at 0.62-Å resolution reveals structural microheterogeneity of protein and solvation. Biophys. J. **84**, 1228–1237 (2003)
9. Du, N., Liu, X.Y., Hew, C.L.: Ice nucleation inhibition. J. Biol. Chem. **278**, 36000–36004 (2003)
10. Liu, X.Y., Du, N.: Zero-sized effect of nano-particles and inverse homogeneous nucleation: principles of freezing and antifreeze. J. Biol. Chem. **279**, 6124 – 6131 (2004)
11. Jessen, T.H., Komiyama, N.H., Tame, J., Pagnier, J., Shih, D., Luisi, B., Fermi, G., Nagai, K.: Production of human hemoglobin in *Escherichia coli* using cleavable fusion protein expression vector. Methods Enzymol. **231**, 347–364 (1994)
12. Schwan, H.P.: Determination of biological impedances. In: Nastuk, W. (ed.) Physical techniques in biological research, vol VIB, pp. 323–407. Academic, New York (1963)
13. Raicu, V., Saibara, T., Irimajiri, A.: Dielectric properties of rat liver in vivo: a noninvasive approach using an open-ended coaxial probe at audio/radio frequencies. Bioelectrochem. Bioenerg. **47**, 325 – 332 (1998)
14. Solovyova, A., Schuck, P., Costenaro, L., Ebel, C.: Non-ideality by sedimentation velocity of malate dehydrogenase in complex solvents. Biophys. J. **81**, 1868–1880 (2001)

15. Bordi, F., Cametti, C., Colby, R.H.: Dielectric spectroscopy and conductivity of polyelectrolyte solutions. J. Phys.: Condens. Matter **16**, 1423–1463 (2004)
16. Grant, E.H., South, G.P.: Dielectric dispersion in aqueous solutions of oxyhaemoglobin and carboxyhaemoglobin. Biochem. J. **122**, 691–699 (1971)
17. Ananthanarayanan, V.S., Slaughter, D., Hew, C.L.: Antifreeze proteins from the ocean pout, *macrozoarces americanus:* circular dichroism spectral studies on the native and denatured states. Biochim. Biophys. Acta **870**, 154–159 (1986)
18. Schrag, J.D., Cheng, C.-H.C., Panico, M., Morris, H.R., DeVries, A.L.: Primary and secondary structure of antifreeze peptides from arctic and antarctic zoarcid fishes. Biochim. Biophys. Acta **915**, 357–370 (1987)
19. Garcia-Arribas, O., Mateo, R., Tomczac, M.M., Davies, P.L., Mateu, M.G.: Thermodynamic stability of a cold-adapted protein, Type III Antifreeze Protein, and energetic contribution of salt bridges. Protein Sci. **16**, 227 – 238 (2007)
20. Grant, E.H., Mitton, B.G.R., South, G.P., Sheppard, R.J.: An investigation by dielectric methods of hydration in myoglobin solutions. Biochem. J. **139**, 375–380 (1974)
21. Budayova-Spano, M., Lafont, S., Astier, J.P., Ebel, C., Veesler, S.: Comparison of solubility and interactions of aprotinin (BPTI) solutions in H_2O and D_2O. J. Cryst. Growth **217**, 311–319 (2000)